FORM PTO 1390 (REV 5-93) US DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY DOCKET NUMBER
2002_0275A

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. §371

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)
NEW 10/070386

International Application No.	**International Filing Date**	**Priority Date Claimed**
PCT/JP00/06104	September 7, 2000	September 7, 1999

Title of Invention
REGULATORY SEQUENCES AND EXPRESSION SYSTEMS FUNCTIONING IN FILAMENTOUS FUNGI

Applicant(s) For DO/EO/US
Manabu WATANABE, Takeshi MURAKAMI

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. §371.

2. [] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. §371.

3. [] This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1).

4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [X] A copy of the International Application as filed (35 U.S.C. §371(c)(2))
   a. [] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau.
   c. [] is not required, as the application was filed in the United States Receiving Office (RO/US)

6. [X] A translation of the International Application into English (35 U.S.C. §371(c)(2)). **ATTACHMENT A**

7. [] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3)).
   a. [] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [] have been transmitted by the International Bureau.
   c. [] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [] have not been made and will not be made.

8. [] A translation of the amendments to the claims under PCT Article 19.

9. [X] An executed oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)). **ATTACHMENT B**

10. [] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)).

Items 11. to 14. below concern other document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98. **ATTACHMENT C**

12. [X] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
ATTACHMENT D

13. [X] A FIRST preliminary amendment. **ATTACHMENT E**
[] A SECOND or SUBSEQUENT preliminary amendment.

14. [X] Other items or information: - Cover Letter with 1 deposit receipt - **ATTACHMENT F**

THE COMMISSIONER IS AUTHORIZED TO CHARGE ANY DEFICIENCY IN THE FEE FOR THIS PAPER TO DEPOSIT ACCOUNT NO. 23-0975.

U.S. APPLICATION NO. (if known, see 37 CFR 1.5) NEW 10/070380	INTERNATIONAL APPLICATION NO. PCT/JP00/06104	ATTORNEY'S DOCKET NO. 2002_0275A

15. [X] The following fees are submitted	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):		
Neither international preliminary examination fee nor international search fee paid to USPTO and International Search Report not prepared by the EPO or JPO $1040.00		
International Search Report has been prepared by the EPO or JPO ..... $ 890.00		
International preliminary examination fee not paid to USPTO but international search paid to USPTO ..... $ 740.00		
International preliminary examination fee paid to USPTO but claims did not satisfy provisions of PCT Article 33(1)-(4) ..... $ 690.00		
International preliminary examination fee paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... $ 100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$890.00	
Surcharge of $130.00 for furnishing the oath or declaration later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	22 -20 =	2	X $18.00	$36.00	
Independent Claims	- 3 =		X $84.00	$	
Multiple dependent claim(s) (if applicable)			+ $280.00	$	
TOTAL OF ABOVE CALCULATIONS =				$926.00	
[] Small Entity Status is hereby asserted. Above fees are reduced by 1/2.				$	
SUBTOTAL =				$926.00	
Processing fee of $130.00 for furnishing the English translation later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$926.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40 per property +				$40.00	
TOTAL FEES ENCLOSED =				$966.00	
				Amount to be refunded	$
				Amount to be charged	$

a. [X] A check in the amount of $966.00 to cover the above fees is enclosed. A duplicate copy of this form is enclosed.

b. [] Please charge my Deposit Account No. 23-0975 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-0975.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

19. CORRESPONDENCE ADDRESS

000513

PATENT TRADEMARK OFFICE

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March 6, 2002

[CHECK NO. 49177]

[2002_0275A]

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	:	**Confirmation No. 2874**
Manabu WATANABE et al.	:	Docket No. 2002-0275A
Serial No. 10/070,38 6	:	Group Art Unit Not Yet Assigned
Filed March 6, 2002	:	Examiner Not Yet Assigned
REGULATORY SEQUENCES AND EXPRESSION SYSTEMS FUNCTIONING IN FILAMENTOUS FUNGI	:	THE COMMISSIONER IS AUTHORIZED TO CHARGE ANY DEFICIENCY IN THE FEES FOR THIS PAPER TO DEPOSIT ACCOUNT NO. 23-0975

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents,
Washington, D.C. 20231

Sir:

Responsive to the Notice dated May 7, 2002, please amend the above-identified application as follows:

In the Specification:

Please replace the Sequence Listing of record with the attached substitute Sequence Listing.

REMARKS

The foregoing amendments are presented to place the application in compliance with the sequence rules under 37 CFR 1.821-1.825.

Applicants have submitted a revised Sequence Listing in both paper and computer readable form as required by 37 C.F.R. 1.821(c) and (e). Amendments directing its entry into the specification have also been incorporated herein. The content of the paper and computer readable copies are the same and no new matter has been added.

In view of the foregoing, it is believed that each requirement set forth in the Notice has been satisfied, and that the application is now in compliance with the sequence rules under 37 CFR 1.821-1.825. Accordingly, favorable examination on the merits is respectfully requested.

A copy of the English translation of the International Preliminary Examination Report is also enclosed to complete the PTO file, since the Notice of Missing Requirements does not confirm receipt thereof.

Respectfully submitted,

Manabu WATANABE et al.

By: _____

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May 21, 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of :

Manabu WATANABE et al. : **Attn: BOX PCT**

Serial No. NEW : **Docket No. 2002_0275A**

Filed March 6, 2002 :

REGULATORY SEQUENCES AND EXPRESSION SYSTEMS FUNCTIONING IN FILAMENTOUS FUNGI
[Corresponding to PCT/JP00/06104
Filed September 7, 2000]

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents,
Washington, DC 20231

Sir:

Prior to calculating the filing fee, please amend the above-identified application as follows:

IN THE SPECIFICATION

Page 1, after the title please insert the following sentence:

This application is a 371 of PCT/JP00/06104 filed September 7, 2000.

IN THE CLAIMS

Please amend the claims as follows:

11. **(Amended)** An expression vector comprising the promoter or the fragment thereof according to claim 1.

12. **(Amended)** An expression vector comprising the terminator or the fragment thereof of claim 6.

ATTACHMENT E

13. **(Amended)** An expression vector comprising a promoter comprising a nucleotide sequence selected from the group consisting of the following sequences, and a fragment thereof having promoter activity:

(a) a nucleotide sequence of SEQ ID NO: 1,

(b) a nucleotide sequence that has at least 70% homology to the sequence of SEQ ID NO: 1 and has promoter activity,

(c) a modified nucleotide sequence of SEQ ID NO: 1 that has one or more modifications selected from a substitution, a deletion, an addition and an insertion and has promoter activity, and

(d) a nucleotide sequence that hybridizes with a nucleotide sequence of SEQ ID NO: 1 under stringent conditions and has promoter activity, together with a terminator comprising a nucleotide sequence selected from the group consisting of the following sequences, and a fragment thereof having terminator activity:

(e) a nucleotide sequence of SEQ ID NO: 2,

(f) a nucleotide sequence that has at least 70% homology to the nucleotide sequence of SEQ ID NO: 2 and has terminator activity,

(g) a modified nucleotide sequence of SEQ ID NO: 2 that has one or more modifications selected from a substitution, a deletion, an addition and an insertion and having terminator activity, and

(h) a nucleotide sequence that hybridizes with a nucleotide sequence of SEQ ID NO: 2 under stringent conditions and has terminator activity.

15. **(Amended)** The expression vector according to claim 11, which further comprises a nucleotide sequence encoding a protein of interest, wherein the nucleotide sequence is operably linked to the promoter and/or the terminator.

16. **(Amended)** A host transformed with the expression vector of claim 15.

18. **(Amended)** A method for producing a protein of interest, which comprises culturing the host of claim 16 and collecting the protein of interest from the culture medium.

Please add the following new claims:

19. **(New)** The expression vector according to claim 13, which further comprises a nucleotide sequence encoding a protein of interest, wherein the nucleotide sequence is operably linked to the promoter and/or the terminator.

20. **(New)** A host transformed with the expression vector of claim 19.

21. **(New)** The host according to claim 20, wherein the host is Mycelia sterilia.

22. **(New)** A method for producing a protein of interest, which comprises culturing the host of claim 20 and collecting the protein of interest from the culture medium.

REMARKS

The specification has been revised to reflect the national stage status. The claims has been amended to remove the multiple dependencies, in order to eliminate the improper multiple dependencies and to reduce the PTO filing fee.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached pages are captioned "**Version with markings to show changes made**".

Favorable action on the merits is solicited.

Respectfully submitted,

Manabu WATANABE et al.

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March 6, 2002

REGULATORY SEQUENCES AND EXPRESSION SYSTEMS FUNCTIONING IN FILAMENTOUS FUNGI

this application is a 371 of PCT/JP00/06104 filed September 7, 2000.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to promoters and terminators which function in filamentous fungi, expression vectors comprising the same, and hosts transformed by said vectors.

Description of the Related Art

The filamentous fungus strain PF1022 (Mycelia sterilia) (FERM BP-2671) produces substance PF1022 which is a 24-membered cyclic depsipeptide having a vermifugal activity. This strain has been classified into Agonomycetes since it forms no sexual or asexual organ (Japanese Patent Application Laid-open No. 35796/1991).

On the other hand, a transformant of the strain PF1022 has been obtained by introducing a plasmid in which the TAKA-amylase gene derived from Aspergillus oryzae is ligated along with a drug resistance gene into the strain PF1022 (WO97/00944).

However, the regulatory DNA sequence of the TAKA-amylase gene derived from Aspergillus oryzae reported in WO97/00944 is a regulatory DNA sequence derived from a strain of heterologous species. Moreover, genetic characteristics of Mycelia sterilia have not sufficiently been revealed and the condition to satisfy expression vector systems has not been elucidated. Accordingly, it is not clear whether gene expression in a transformant having a regulatory sequence derived from a strain of heterologous species can be coordinately regulated with the expression of an endogenous gene in the strain PF1022. Furthermore, since the strain PF1022 belongs to Agonomycetes, it is also not clear whether conventional regulatory DNA sequences used in microorganisms other than genus Aspergillus, such as genus Trichoderma, genus Fusarium, genus Neurospora or the like, can be appropriately expressed.

Accordingly, a regulatory sequence and an expression vector system which stably function in Mycelia sterilia, and establishment of technology for producing useful substances in

VERSION WITH MARKINGS TO SHOW CHANGES MADE

11. **(Amended)** An expression vector comprising the promoter or the fragment thereof according to [any one of claims 1 to 5] claim 1.

12. **(Amended)** An expression vector comprising the terminator or the fragment thereof of [any one of claims 6 to 10] claim 6.

13. **(Amended)** An expression vector comprising [the promoter or the fragment thereof of any one of claims 1 to 5 and the terminator or the fragment thereof of any one of claims 6 to 10] a promoter comprising a nucleotide sequence selected from the group consisting of the following sequences, and a fragment thereof having promoter activity:

(a) a nucleotide sequence of SEQ ID NO: 1,

(b) a nucleotide sequence that has at least 70% homology to the sequence of SEQ ID NO: 1 and has promoter activity,

(c) a modified nucleotide sequence of SEQ ID NO: 1 that has one or more modifications selected from a substitution, a deletion, an addition and an insertion and has promoter activity, and

(d) a nucleotide sequence that hybridizes with a nucleotide sequence of SEQ ID NO: 1 under stringent conditions and has promoter activity, together with a terminator comprising a nucleotide sequence selected from the group consisting of the following sequences, and a fragment thereof having terminator activity:

(e) a nucleotide sequence of SEQ ID NO: 2,

(f) a nucleotide sequence that has at least 70% homology to the nucleotide sequence of SEQ ID NO: 2 and has terminator activity,

(g) a modified nucleotide sequence of SEQ ID NO: 2 that has one or more modifications selected from a substitution, a deletion, an addition and an insertion and having terminator activity, and

(h) a nucleotide sequence that hybridizes with a nucleotide sequence of SEQ ID NO: 2 under stringent conditions and has terminator activity.

VERSION WITH MARKINGS TO SHOW CHANGES MADE

15. **(Amended)** The expression vector according to [any one of claims 11 to 14] claim 11, which further comprises a nucleotide sequence encoding a protein of interest, wherein the nucleotide sequence is operably linked to the promoter and/or the terminator.

16. **(Amended)** A host transformed with the expression vector of [any one of claims 11 to 15] claim 15.

18. **(Amended)** A method for producing a protein of interest, which comprises culturing the host of claim 16 [or 17] and collecting the protein of interest from the culture medium.

(12)特許協力条約に基づいて公開された国際出願

(19) 世界知的所有権機関
国際事務局

(43) 国際公開日
2001 年3 月15 日 (15.03.2001)

PCT

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(81) 指定国 (国内): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

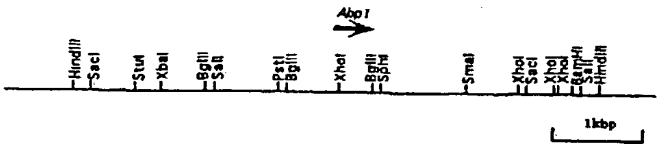
(84) 指定国 (広域): ARIPO 特許 (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), ユーラシア特許 (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), ヨーロッパ特許 (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI 特許 (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

添付公開書類:
— 国際調査報告書

２文字コード及び他の略語については、定期発行される各PCTガゼットの巻頭に掲載されている「コードと略語のガイダンスノート」を参照。

(54) Title: REGULATORY SEQUENCES FUNCTIONING IN FILAMENTOUS FUNGI

(54) 発明の名称: 糸状菌において機能する制御配列および発現系



(57) Abstract: A promoter and a terminator functioning synchronously with the expression of an endogenous gene in filamentous fungi belonging to *Agonomycetales* (in particular, *Mycelia sterilia*). The above promoter contains the nucleotide sequence represented by SEQ ID NO:1 and its homolog. The above terminator contains the nucleotide sequence represented by SEQ ID NO:2 and its homolog. Further, an expression vector highly expressing a target protein in filamentous fungi; a transformed filamentous fungus capable of producing the target protein at a high yield; and a process for producing the target protein in the transformed filamentous fungus; are provided.

[続葉有]

WO 01/18219 A1

REGULATORY SEQUENCES AND EXPRESSION SYSTEMS FUNCTIONING IN FILAMENTOUS FUNGI

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to promoters and terminators which function in filamentous fungi, expression vectors comprising the same, and hosts transformed by said vectors.

Description of the Related Art

The filamentous fungus strain PF1022 (Mycelia sterilia) (FERM BP-2671) produces substance PF1022 which is a 24-membered cyclic depsipeptide having a vermifugal activity. This strain has been classified into Agonomycetes since it forms no sexual or asexual organ (Japanese Patent Application Laid-open No. 35796/1991).

On the other hand, a transformant of the strain PF1022 has been obtained by introducing a plasmid in which the TAKA-amylase gene derived from Aspergillus oryzae is ligated along with a drug resistance gene into the strain PF1022 (WO97/00944).

However, the regulatory DNA sequence of the TAKA-amylase gene derived from Aspergillus oryzae reported in WO97/00944 is a regulatory DNA sequence derived from a strain of heterologous species. Moreover, genetic characteristics of Mycelia sterilia have not sufficiently been revealed and the condition to satisfy expression vector systems has not been elucidated. Accordingly, it is not clear whether gene expression in a transformant having a regulatory sequence derived from a strain of heterologous species can be coordinately regulated with the expression of an endogenous gene in the strain PF1022. Furthermore, since the strain PF1022 belongs to Agonomycetes, it is also not clear whether conventional regulatory DNA sequences used in microorganisms other than genus Aspergillus, such as genus Trichoderma, genus Fusarium, genus Neurospora or the like, can be appropriately expressed.

Accordingly, a regulatory sequence and an expression vector system which stably function in Mycelia sterilia, and establishment of technology for producing useful substances in

Mycelia sterilia using the same are highly desired.

SUMMARY OF THE INVENTION

An objective of the present invention is to provide a regulatory sequence that functionally coordinates with the expression of an endogenous gene in a filamentous fungus that belongs to Agonomycetes, particularly in Mycelia sterilia.

Another objective of the present invention is to provide an expression vector that highly expresses a protein of interest in a filamentous fungus that belongs to Agonomycetes, particularly in Mycelia sterilia.

Still another objective of the present invention is to provide a process of producing a protein of interest in a filamentous fungus that belongs to Agonomycetes, particularly in Mycelia sterilia.

The present inventors succeeded in isolating and identifying a highly expressing gene (Abp1 gene) and its regulatory DNA sequences.

The present inventors also succeeded in constructing an expression vector for gene expression using the regulatory DNA sequences thus obtained, introducing this vector into a PF1022-producing microorganism to obtain a transformant, and highly expressing a gene of interest ligated downstream of the promoter of this transformant without making the gene malfunction.

A promoter according to the present invention comprises a nucleotide sequence selected from the group consisting of the following sequences, and a fragment thereof having promoter activity:

(a) a nucleotide sequence of SEQ ID NO: 1,

(b) a nucleotide sequence that has at least 70% homology to the sequence of SEQ ID NO: 1 and has promoter activity,

(c) a modified nucleotide sequence of SEQ ID NO: 1 that has one or more modifications selected from a substitution, a deletion, an addition and an insertion and has promoter activity, and

(d) a nucleotide sequence that hybridizes with a nucleotide

sequence of SEQ ID NO: 1 under stringent conditions and has promoter activity.

A terminator according to the present invention comprises a nucleotide sequence selected from the group consisting of the following sequences, and a fragment thereof having terminator activity:

(e) a nucleotide sequence of SEQ ID NO: 2,

(f) a nucleotide sequence that has at least 70% homology to the nucleotide sequence of SEQ ID NO: 2 and has terminator activity,

(g) a modified nucleotide sequence of SEQ ID NO: 2 that has one or more modifications selected from a substitution, a deletion, an addition and an insertion and having terminator activity, and

(h) a nucleotide sequence that hybridizes with a nucleotide sequence of SEQ ID NO: 2 under stringent conditions and has terminator activity.

An expression vector of the present invention comprises either one or both of the abovementioned promoter or a fragment thereof and the above-mentioned terminator or a fragment thereof.

A transformed host according to the present invention is a host transformed with the abovementioned expression vector.

A process for producing a substance of interest according to the present invention comprises culturing the above-mentioned transformed host and collecting the protein of interest from the culture medium.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a restriction map of a 6 kb HindIII fragment comprising the Abp1 gene.

Figure 2 shows the construction and restriction map for pABPd.

DETAILED DESCRIPTION OF THE INVENTION

Deposition of microorganism

The strain PF1022 was deposited with the National Institute of Bioscience and Human-Technology Agency of Industrial Science

and Technology, the Ministry of International Trade and Industry (1-3 Higashi 1-Chome, Tsukuba City, Ibaraki Prefecture, Japan), dated January 24, 1989. The accession number is FERM BP-2671.

Regulatory sequences

According to the present invention, there are provided regulatory sequences, namely a promoter and a terminator, which function in a PF1022-producing microorganism.

Sequence (b) can have preferably at least 80%, more preferably at least 90%, or most preferably at least 95% homology to the nucleotide sequence of SEQ ID NO: 1.

Sequence (f) can have preferably at least 80%, more preferably at least 90%, or most preferably at least 95% homology to the nucleotide sequence of SEQ ID NO: 2.

In sequences (c) and (g), the number of modifications can be, for example, one to dozens.

In sequences (c) and (g), if multiple modifications are introduced, said modifications may be the same or different.

In sequences (d) and (h), the term "stringent conditions" means that a membrane after hybridization is washed at a high temperature in a solution of low salt concentration, for example, at 60°C for 15 minutes in a solution of 0.5 x SSC concentration (1 x SSC: 15 mM trisodium citrate, 150 mM sodium chloride), more preferably at 60°C for 15 minutes in solution of 0.5 x SSC concentration and 0.1% SDS.

The length of a fragment having promoter activity can be at least 600 base pairs, preferably at least 800 base pairs, more preferably at least 1000 base pairs, and most preferably at least 1200 base pairs.

The length of a fragment having terminator activity can be at least 400 base pairs, preferably at least 600 base pairs, more preferably at least 800 base pairs, and most preferably at least 1000 base pairs.

Whether sequences (b), (c) and (d) and fragments having promoter activity "have the promoter activity" or not can be evaluated, for example, by constructing an expression vector as described in Example 3, expressing a heterologous gene in a host as described in Example 4, and detecting the production of a

heterologous protein.

Whether sequences (f), (g) and (h) and fragments having terminator activity "have the terminator activity" or not can be evaluated, for example, by constructing an expression vector as described in Example 3, expressing a heterologous gene in a host as described in Example 4, and detecting the production of a heterologous protein.

A promoter and a terminator according to the present invention can function in a filamentous fungus that is classified into Agonomycetes, particularly a microorganism that belongs to Mycelia, more specifically a microorganism that belongs to Mycelia sterilia.

A promoter and a terminator according to the present invention can function in a PF1022-producing microorganism. An example of the PF1022-producing microorganism is a filamentous fungus that is classified into Agonomycetes producing the substance PF1022.

A promoter and a terminator according to the present invention can be obtained, for example, as follows.

mRNAs of the strain PF1022 are isolated from the cells during substance PF1022 production and cDNAs are synthesized using the isolated mRNAs as a template. The cDNAs are randomly sampled and the DNA sequences of the sampled cDNAs are analyzed to isolate cDNAs derived from a highly expressing gene, namely the Abp1 gene.

A genomic DNA is isolated from the strain PF1022 and cleaved with appropriate restriction enzymes, and a library comprising the genomic DNA of the PF1022-producing microorganism is constructed using a phage vector or a plasmid vector.

The entire length of the Abp1 gene is cloned from the genomic DNA library derived from the strain PF1022 thus prepared using a translation region encoding the Abp1 gene as a probe. The isolated genomic DNA and the DNA sequence of the above-mentioned cDNAs are compared and promoter and terminator sites of this gene are determined to identify a promoter and a terminator.

Expression vectors

The present invention provides an expression vector comprising a regulatory sequence which functions in a PF1022-producing microorganism.

The procedure and method for constructing an expression vector according to the present invention can be any of those commonly used in the field of genetic engineering.

Examples of the expression vector as used herein include vectors which can be incorporated into a host chromosome DNA and vectors having a self-replicable autonomous replication sequence which can be present as a plasmid in a host cell, for example, pUC vectors (e.g., pUC18 and pUC118), pBluescript vectors (e.g., pBluescriptII KS+), and plasmids such as pBR322 plasmid. One or more of copies of the gene can be present in a host cell.

An expression vector according to the present invention in the first embodiment comprises a promoter and/or a terminator according to the present invention, and if appropriate, a gene marker and/or other regulatory sequences. Thus, an expression vector comprising either one or both of the promoter and the terminator according to the present invention is within the scope of the present invention.

An expression vector that at least comprises the promoter according to the present invention can comprise a terminator other than the terminator according to the present invention.

An expression vector that at least comprises the terminator according to the present invention can comprise a promoter other than the promoter according to the present invention.

A gene marker can be introduced, for example, by introducing an appropriate restriction enzyme cleaving site into a regulatory sequence of the present invention by the PCR method, inserting this into a plasmid vector, and ligating a selective marker gene such as a drug resistance gene and/or a gene complementing a nutritional requirement.

A gene marker can be appropriately selected depending on the technique for selecting a transformant. For example, a gene encoding drug resistance or a gene complementing a nutritional requirement can be used. Examples of the drug resistance gene

include genes conferring resistance to destomycin, benomyl, oligomycin, hygromycin, G418, bleomycin, bialaphos, blastcidin S, phleomycin, phosphinothricin, ampicillin, and kanamycin. Examples of the gene complementing a nutritional requirement include amdS, pyrG, argB, trpC, niaD, TRP1, LEU2, URA3 and the like.

An expression vector according to the present invention in the second embodiment can further comprise a nucleotide sequence encoding a protein of interest, which is operably linked to a regulatory sequence.

The ligation to a regulatory sequence can be carried out, for example, according to an ordinary method by inserting a translation region of a gene encoding a protein of interest (gene of interest) downstream of a promoter in the right direction. In this case, the protein can be expressed as a fusion protein by ligating the gene of interest with a foreign gene encoding a translation region of another protein. In the present specification, the term "gene of interest" means a given gene to be subjected to expression and can be either a heterologous gene or a homologous gene. The gene of interest can be, for example, a gene selected from a group related to the production of substance PF1022.

Production of transformant and protein of interest

The present invention provides a host transformed with the above-mentioned expression vector. A host to be used in the present invention is not particularly restricted and any microorganism which can be used as a host for genetic recombination, for example, a filamentous fungus, preferably a filamentous fungus classified into Agonomycetes, more preferably a microorganism that belongs to Mycelia, most preferably a microorganism that belongs to Mycelia sterilia, can be used. Examples of the host to be used in the present invention include a PF1022-producing microorganism, preferably a filamentous fungus producing substance PF1022, more preferably the strain PF1022 (FERM BP-2671) producing substance PF1022.

A recombinant vector for the gene expression can be introduced into a host by an ordinary method. Examples of the

method for the introduction include the electroporation method, the polyethylene glycol method, the aglobacterium method, the lithium method, and the calcium chloride method. A method suitable to each host cell can be selected. The polyethylene glycol method is preferable when a PF1022-producing microorganism is used as a host.

The present invention provides a process for producing a protein of interest including a step of culturing the above-mentioned transformant.

A transformant can be cultured according to an ordinary method by using a medium, culture conditions and the like, which are appropriately selected. Conventional components can be used for a medium. As a carbon source, glucose, sucrose, cellulose, starch syrup, dextrin, starch, glycerol, molasses, animal and vegetable oils, and the like can be used. As a nitrogen source, soybean powder, wheat germ, pharma media, cornsteep liquor, cotton seed lees, bouillon, peptone, polypeptone, malt extract, ammonium sulfate, sodium nitrate, urea, and the like can be used. If necessary, sodium, potassium, calcium, magnesium, cobalt, chlorine, phosphoric acid, sulfuric acid, and other inorganic salts that can produce ions, such as potassium chloride, calcium carbonate, dipotassium hydrogenphosphate, magnesium sulfate, monopotassium phosphate, zinc sulfate, manganese sulfate, and copper sulfate, can be effectively added. If necessary, various vitamins such as thiamine (e.g., thiamine hydrochloride), amino acids such as glutamic acid (e.g., sodium glutamate) and asparagine (e.g., DL-asparagine), trace nutrients such as nucleotides, and selective drugs such as antibiotics can be added. Further, organic and inorganic substances to promote the growth of microorganisms and enhance the production of cyclic depsipeptide can be appropriately added.

The cultivation can be carried out in a liquid medium by a culture method under an aerobic condition, a shaking culture method, an agitation culture method with aeration, or a submerged culture method. The pH of the medium is, for example, about 6 to 8. The cultivation can be carried out at a normal temperature, such as 14°C to 40°C, preferably 26°C to 37°C, for about 2 to 25

days.

Furthermore, in a process of producing a protein of interest according to the present invention, the protein of interest, namely the gene expression product, can be obtained from the culture of transformed cells. The protein of interest can be extracted from the culture (e.g., by mashing, and crushing under pressure), recovered (e.g., by filtration, and centrifugation), and purified (e.g., by salting out, and solvent precipitation). Furthermore, in these steps, a protease inhibitor such as phenylmethylsulfonyl fluoride (PMSF), benzamidine and leupeptin can be added, if necessary.

EXAMPLE

The present invention is further illustrated by the following examples that are not intended as a limitation of the invention.

Example 1: Search for a highly expressing gene by random sequencing of cDNA

In order to search for a highly expressing gene in a substance PF1022-producing microorganism, cDNAs derived from the substance PF1022-producing microorganism were randomly cloned, DNA sequences of the products were compared, and a gene which was highly expressed was isolated and identified.

(1) Preparation of cDNA derived from a substance PF1022-producing microorganism

The strain PF1022 (FERM BP-2671) was cultured in a production medium (2.0% glucose, 5.0% starch, 0.8% wheat germ, 1.3% soybean cake, 0.38% meat extract, 0.13% sodium chloride, and 0.15% calcium carbonate; pH 7.0 before sterilization; see Example 4 in WO97/00944) at 26°C for 4 days, and the resulting cells were recovered by centrifugation (3000 rpm, 10 minutes). The cells were washed with purified water, frozen at -80°C, and then smashed with a blender (AM-3, Nippon Seiki Industry Co., Ltd.) under the presence of liquid nitrogen. The resulting smashed cells were suspended in a denaturation solution (4 M guanidine thiocyanate, 25 mM trisodium citrate, 0.5% sodium N-lauryl sarcosinate, 0.1M mercapto ethanol), the suspension was

stirred at room temperature for 5 minutes and then neutralized with 2 M sodium citrate (pH 4.5), TE-saturated phenol was added, and the resulting admixture was further stirred. Chloroform-isoamyl alcohol (24:1) was added to the admixture and after stirring, the cell component denatured with phenol was isolated by centrifugation. The upper layer (aqueous layer) was recovered, and the nucleic acid was precipitated with isopropanol. The precipitate was dissolved in a TE solution (10 mM tris-hydrochloric acid (pH 8.0), 1 mM EDTA) to a nucleic acid concentration of 1 mg/ml and then precipitated with 2.5 M lithium chloride (5°C, 2 hours). The resulting precipitate was recovered by centrifugation, washed with 70% ethanol and redissolved in a TE solution to obtain the total RNA fraction.

From this total RNA fraction, mRNA was purified using an mRNA purification kit (Amersham Pharmacia Biotech). Further, cDNA was synthesized using this mRNA as a template using a Timesaver cDNA synthesis kit (Amersham Pharmacia Biotech).

(2) Random Sequencing of cDNA

The cDNA prepared in Example 1 (1) was cleaved with EcoRI, after which ligation to pUC18 treated with alkaline phosphatase was carried out using a DNA ligation kit Ver. 2 (Takara Shuzo Co., Ltd.). Transformation was carried out with E.coli JM109 strain and various transformed colonies were cultured in an LB medium (1% polypeptone, 0.5% yeast extract, 1% sodium chloride) supplemented with ampicillin. Plasmids from these transformants were purified using a Flexi Prep kit (Amersham Pharmacia Biotech).

Forty kinds of plasmids prepared as described above were subjected to an ALF DNA sequencer II (Amersham Pharmacia Biotech) and the DNA sequences of inserted fragments were analyzed. The sequence gel used was Long Ranger (FMC Co.) and the sequencing reaction was carried out using an Autoread sequencing kit (Amersham Pharmacia Biotech).

As a result, ten kinds of clones were found to have an identical DNA sequence. The cloned gene was named Abp1, and the promoter and the terminator of this gene were to be cloned from the genomic DNA.

(3) Isolation of genomic DNA of substance PF1022-producing

microorganism

The genomic DNA of the strain PF1022 was isolated according to the method of Horiuchi et al. (H. Horiuchi et al., J. Bacteriol., 170, 272-278, 1988). More specifically, cells of substance PF1022-producing strain (FERM BP-2671) were cultured for 2 days in a seed medium (2.0% soluble starch, 1.0% glucose, 0.5% polypeptone, 0.6% wheat germ, 0.3% yeast extract, 0.2% soybean cake, and 0.2% calcium carbonate; pH 7.0 before sterilization; see Example 1 in WO97/00944) and the cells were recovered by centrifugation (3500 rpm, 10 minutes). The cells thus obtained were lyophilized and then suspended in a TE solution, treated in a 3% SDS solution at 60°C for 30 minutes, and then subjected to TE-saturated phenol extraction to remove the cell residue. The extract was precipitated with ethanol and treated with ribonuclease A (Sigma) and proteinase K (Wako Pure Chemical Industries, Ltd.), and then the nucleic acid was precipitated with 12% polyethylene glycol 6000. The precipitate was subjected to TE-saturated phenol extraction and ethanol precipitation, and the resulting precipitate was dissolved in a TE solution to obtain the genomic DNA.

(4) Construction of genomic DNA library of substance PF1022-producing microorganism

The genomic DNA derived from the substance PF1022-producing microorganism prepared in Example 1 (3) was partially digested with Sau3AI. The product was ligated to the BamHI arm of a phage vector, λEMBL3 cloning kit (Stratagene Co.) using T4 ligase (Ligation Kit Ver. 2; Takara Shuzo Co., Ltd.). After ethanol precipitation, the precipitate was dissolved in a TE solution. The entire ligated mixture was used to infect E.coli LE392 strain using a Gigapack II Plus Packaging kit (Stratagene Co.) to form a phage plaque. The 1.3×10^4 (2.6×10^4 PFU/ml) phage library obtained by this method was used for cloning of the Abp1 gene.

(5) Cloning of the Abp1 gene from the genomic DNA derived from substance PF1022-producing microorganism

A probe to be used was prepared by amplifying the translation region of the Abp1 gene by the PCR method. The PCR

was carried out using the genomic DNA prepared in Example 1 (3) as a template and synthesis primers 8-73U and 8-73R according to a LETS GO PCR kit (SAWADY Technology). The PCR reaction for amplification was conducted by repeating 25 cycles of 30 seconds at 94°C, 30 seconds at 50°C, and 90 seconds at 72°C. DNA sequences of the 8-73U and 8-73R are as follows:

8-73U: CACAAACCAGGAACTCTTTC (SEQ ID NO: 7)

8-73R: GACATGTGGAAACCACATTTTG (SEQ ID NO: 8)

The PCR product thus obtained was labeled using an ECL Direct System (Amersham Pharmacia Biotech). The phage plaque prepared in Example 1 (4) was transferred to a Hibond N+ nylon transfer membrane (Amersham Pharmacia Biotech), and after alkaline denaturation, the membrane was washed with 5-fold concentration SSC (SSC: 15 mM trisodium citrate, 150 mM sodium chloride), and dried to immobilize the DNA. According to the kit protocol, prehybridization (42°C) was carried out for 1 hour, after which the previously labeled probe was added and hybridization was carried out at 42°C for 16 hours. The probe was washed according to the kit protocol above. The nylon membrane used with the washed probe was immersed for one minute in a detection solution, and was then photosensitized on a medical X-ray film (Fuji Photo Film Co., Ltd.) to obtain one positive clone. Southern blot analysis of this clone showed that a HindIII fragment of at least 6 kb was identical with the restriction enzyme fragment of the genomic DNA. Figure 1 shows the restriction map of this HindIII fragment. The HindIII fragment was subcloned in pUC119 to obtain pRQHin/119 for the following experiment.

Example 2: Determination of DNA sequences of promoter and terminator of Abp1 gene

A template for DNA sequence analysis was prepared by digesting pRQHin/119 with SalI and SmaI and ligating the resultant fragment with pUC18 previously digested with the same restriction enzymes. The DNA sequence analysis was carried out in the same manner as described in Example 1 (2). Next, the DNA sequence thus obtained was compared with that of cDNA obtained in Example 1 (2), and sequences of the promoter and terminator regions of the Abp1 gene were determined. The resulting DNA sequences are shown

in SEQ ID NO: 1 and SEQ ID NO: 2.

Example 3: Construction of expression vector using the expression regulatory region of the Abp1 gene

The promoter region and the terminator region of the Abp1 gene were amplified by the PCR method using pRQHin/119 as a template. The PCR method was carried out using PCR Super Mix High Fidelity (Lifetech Oriental Co., Ltd.) with primers ABP-Neco and ABP-Nbam for promoter amplification and ABP-Cbam and ABP-Cxba for terminator amplification. The amplification reaction was conducted by repeating 25 cycles of 30 seconds at 94°c, 30 seconds at 50°c and 90 seconds at 72°c. The DNA sequences of ABP-Neco, ABP-Nbam, ABP-Cbam and ABP-Cxba are as follows:

ABP-Neco: GGGGAATTCGTGGGTGGTGATATCATGGC (SEQ ID NO: 3)

ABP-Nbam: GGGGGATCCTTGATGGGTTTTGGG (SEQ ID NO: 4)

ABP-Cbam: GGGGGATCCTAAACTCCCATCTATAGC (SEQ ID NO: 5)

ABP-Cxba: GGGTCTAGACGACTCATTGCAGTGAGTGG (SEQ ID NO: 6)

Each PCR product was purified with a Microspin S-400 column (Amersham Pharmacia Biotech) and precipitated with ethanol, after which the promoter was digested with EcoRI and BamHI, the terminator was digested with BamHI and XbaI, and the resulting fragments were ligated one by one with pBleuscriptII KS+ previously digested with the same enzymes. The product was digested with XbaI, and a destomycin resistance cassette derived from pMKD01 (WO98/03667) was inserted to construct pABPd (Figure 2).

Example 4: Confirmation of ability of expression vector using β-glucuronidase gene

The translation region of the β-glucuronidase (GUS) gene used as a reporter gene was obtained by digesting pLC-GUS (K. Yanai, et al., Biosci. Biotech. Biochem., 60, 472-475, 1996) with BamHI. This fragment was ligated with pABPd which was previously digested with BamHI and treated with alkaline phosphatase to construct plasmid pABPd-G in which the GUS gene was inserted downstream of the Abp1 promoter.

The microorganism producing substance PF1022 (FERM BP-2671) was transformed with pABPd-G according to the method described in Example 1 of WO97/00944. As a result, about three

transformants per 1 μg of DNA were obtained.

The transformants thus obtained were cultured in a liquid using the production medium of Example 1 (1) and the cells were recovered by centrifugation. The resultant cells were disrupted using a Mini-Bead beater (Biospeck Products). Cell debris was removed by centrifugation and the supernatant was measured for GUS activity. The activity was measured by the method described in K. Yanai, et al., Biosci. Biotech. Biochem., 60, 472-475, 1996.

Results in Table 1 evidently confirmed that only the pABPd-G transformants had marked GUS activity. Namely, it was confirmed that the expression vector pABPd effectively functions in the substance PF1022-producing microorganism.

Table 1: GUS Activity of Transformants

	Expression vector	GUS activity (A405/μg protein)
Transformant	pABPd-G	756.9
Transformant	pABPd-G	832.5
Transformant	pABPd	0.0
Host	-	0.0

CLAIMS

1. A promoter comprising a nucleotide sequence selected from the group consisting of the following sequences, and a fragment thereof having promoter activity:

(a) a nucleotide sequence of SEQ ID NO: 1,

(b) a nucleotide sequence that has at least 70% homology to the sequence of SEQ ID NO: 1 and has promoter activity,

(c) a modified nucleotide sequence of SEQ ID NO: 1 that has one or more modifications selected from a substitution, a deletion, an addition and an insertion and has promoter activity, and

(d) a nucleotide sequence that hybridizes with a nucleotide sequence of SEQ ID NO: 1 under stringent conditions and has promoter activity.

2. The promoter according to claim 1, wherein sequence (b) is a nucleotide sequence having at least 80% homology to the nucleotide sequence of SEQ ID NO: 1.

3. The promoter according to claim 1, wherein sequence (b) is a nucleotide sequence having at least 90% homology to the nucleotide sequence of SEQ ID NO: 1.

4. The promoter according to claim 1, which functions in a filamentous fungus that belongs to Agonomycetes.

5. The promoter according to claim 1, wherein the length of the fragment having promoter activity is at least 600 bp.

6. A terminator comprising a nucleotide sequence selected from the group consisting of the following sequences, and a fragment thereof having terminator activity:

(e) a nucleotide sequence of SEQ ID NO: 2,

(f) a nucleotide sequence that has at least 70% homology to the nucleotide sequence of SEQ ID NO: 2 and has terminator activity,

(g) a modified nucleotide sequence of SEQ ID NO: 2 that has one or more modifications selected from a substitution, a deletion, an addition and an insertion and having terminator activity, and
(h) a nucleotide sequence that hybridizes with a nucleotide sequence of SEQ ID NO: 2 under stringent conditions and has terminator activity.

7. The terminator according to claim 6, wherein sequence (f) is a nucleotide sequence having at least 80% homology to the nucleotide sequence of SEQ ID NO: 2.

8. The terminator according to claim 6, wherein sequence (f) is a nucleotide sequence having at least 90% homology to the nucleotide sequence of SEQ ID NO: 2.

9. The terminator according to claim 6, which functions in a filamentous fungus that belongs to Agonomycetes.

10. The terminator according to claim 6, wherein the length of the fragment having terminator activity is at least 400 bp.

11. An expression vector comprising the promoter or the fragment thereof according to any one of claims 1 to 5.

12. An expression vector comprising the terminator or the fragment thereof of any one of claims 6 to 10.

13. An expression vector comprising the promoter or the fragment thereof of any one of claims 1 to 5 and the terminator or the fragment thereof of any one of claims 6 to 10.

14. The expression vector according to claim 13, wherein the expression vector is pABPd.

15. The expression vector according to any one of claims

11 to 14, which further comprises a nucleotide sequence encoding a protein of interest, wherein the nucleotide sequence is operably linked to the promoter and/or the terminator.

16. A host transformed with the expression vector of any one of claims 11 to 15.

17. The host according to claim 16, wherein the host is Mycelia sterilia.

18. A method for producing a protein of interest, which comprises culturing the host of claim 16 or 17 and collecting the protein of interest from the culture medium.

ABSTRACT

The present invention provides a promoter and a terminator which coordinately function with the expression of an endogenous gene in a filamentous fungus that belongs to Agonomycetes, particularly in Mycelia sterilia. A promoter of the present invention comprises the nucleotide sequence of SEQ ID NO: 1 and a homologue thereof. A terminator of the present invention comprises the nucleotide sequence of SEQ ID NO: 2 and a homologue thereof. The present invention further provides an expression vector that highly expresses a protein of interest in a filamentous fungus, a transformed filamentous fungus that highly produces a protein of interest, and a process of producing the protein of interest in the transformed filamentous fungus.

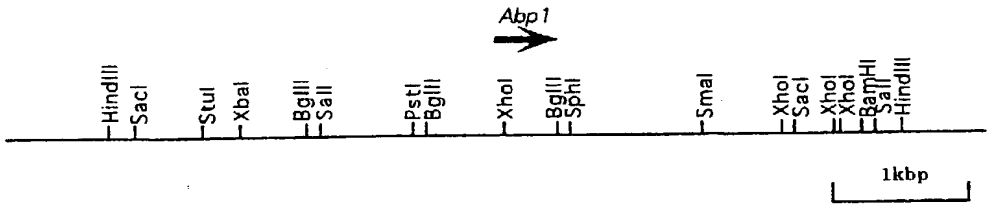

Abp1
HindIII
SacI
StuI
XbaI
BglII
SalI
PstI
BglII
XhoI
BglII
SphI
SmaI
XhoI
SacI
XhoI
XhoI
BamHI
SalI
HindIII
1kbp

FIG. 1

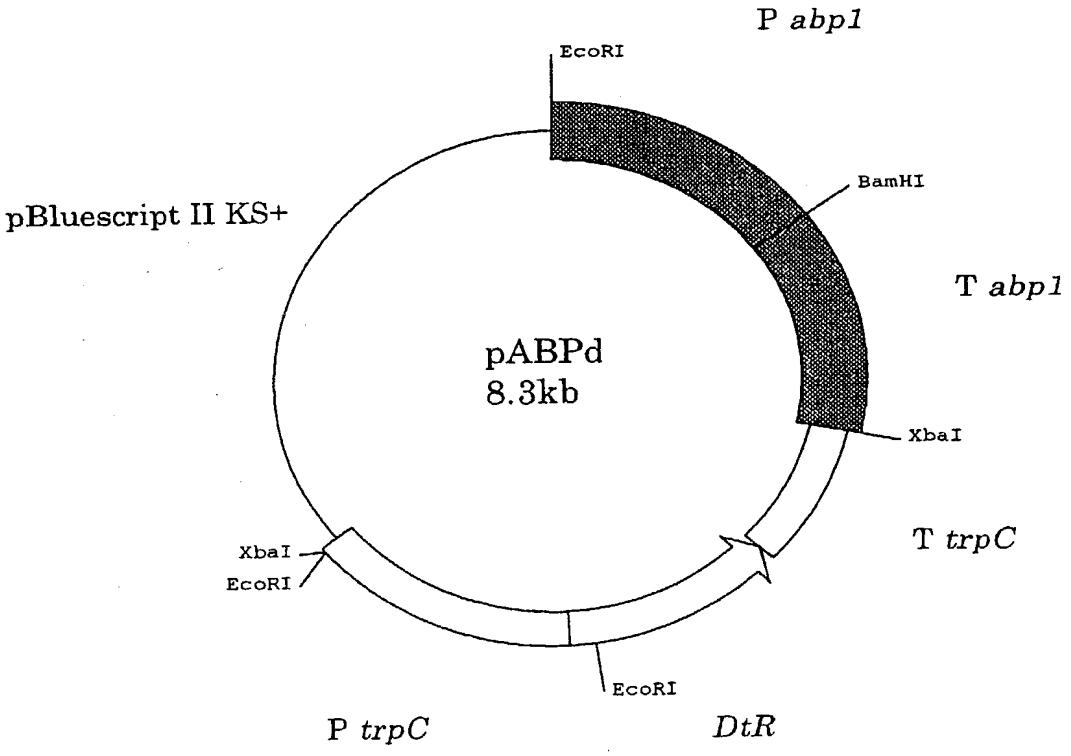

P abp1
EcoRI
BamHI
pBluescript II KS+
T abp1
pABPd
8.3kb
XbaI
T trpC
XbaI
EcoRI
EcoRI
P trpC
DtR

FIG. 2

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659

Attorney's Ref. No.:

Declaration and Power of Attorney For Patent Application

特許出願宣言書及び委任状

Japanese Language Declaration

日本語宣言書

私は、以下に記名された発明者として、ここに下記の通り宣言する：

As a below named inventor, I hereby declare that:

私の住所、郵便の宛先そして国籍は、私の氏名の後に記載された通りである。

My residence, post office address and citizenship are as stated next to my name.

下記の名称の発明について特許請求範囲に記載され、且つ特許が求められている発明主題に関して、私が最初、最先且つ唯一の発明者である（唯一の氏名が記載されている場合）か、或いは最初、最先且つ共同発明者である（複数の氏名が記載されている場合）と信じている。

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

"REGULATORY SEQUENCES AND EXPRESSION SYSTEMS FUNCTIONING IN FILAMENTOUS FUNGI"

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the specification of which is attached hereto unless the following box is checked:

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であり、且つ、
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☒ was filed on 07/September/2000
as United States Application Number or
PCT International Application Number
PCT/JP00/06104 and was amended on
(if applicable).

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I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

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ATTACHMENT B

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I hereby claim foreign priority under Title 35, United States Code, Section 119 (a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)
外国での先行出願

Priority Not Claimed
優先権主張なし

(Number) (番号)	(Country) (国名)	(Day/Month/Year Filed) (出願年月日)	
11-252851	Japan	07/September/1999	☐
	Japan		☐

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I hereby claim the benefit under Title 35, United States Code, Section 119 (e) of any United States provisional application(s) listed below.

(Application No.) (出願番号)	(Filing Date) (出願日)	(Application No.) (出願番号)	(Filing Date) (出願日)

私は、ここに、下記のいかなる米国出願についても、その米国法典第３５編第１２０条に基づく利益を主張し、又米国を指定するいかなるＰＣＴ国際出願についても、その同第３６５条(ｃ)に基づく利益を主張する。また、本出願の各特許請求の範囲の主題が米国法典第３５編第１１２条第１段に規定された態様で、先行する米国特許出願又はＰＣＴ国際出願に開示されていない場合においては、その先行出願の出願日と本国内出願日またはＰＣＴ国際出願日との間の期間中に入手された情報で、連邦規則法典第３７編規則１．５６に定義された特許性に関わる重要な情報について開示義務があることを承認する。

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s), or 365 (c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of application:

(Application No.) (出願番号)	(Filing Date) (出願日)	(Status: Patented, Pending, Abandoned) (現況：特許許可済、係属中、放棄済)

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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委任状： 私は本出願を審査する手続を行い、且つ米国特許商標庁との全ての業務を遂行するために、記名された発明者として、下記の弁護士及び／または弁理士を任命する。（氏名及び登録番号を記載すること）

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Matthew M. Jacob, Reg. 25,154;
Jeffrey Nolton, Reg. 25,408;

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

Warren M. Cheek, Jr., Reg. 33,367;
Nils E. Pedersen, Reg. 33,145;
Charles R. Watts, Reg. 33,142

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発明者の署名 日付	Inventor's signature Date Manabu Watanabe February 12, 2002
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国籍 日本	Citizenship Japan
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第二共同発明者の署名 日付	Second inventor's signature Date Takeshi Murakami February 12, 2002
住所 日本国,	Residence Odawara-Shi, Kanagawa-Ken, Japan JPX
国籍 日本	Citizenship Japan
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（第三以降の共同発明者についても同様に記載し、署名をすること）	(Supply similar information and signature for third and subsequent joint inventors.)

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